Standard testing guidelines are not yet available for the evaluation of the in vitro fungicidal activity of antifungal agents. In order to be clinically useful, in vitro methods should reliably predict in vivo response to therapy.

As new antifungal agents are introduced for the treatment of infections caused by yeasts and filamentous fungi (moulds), it is important that reliable methods are available for the in vitro testing of both new and established agents. The ultimate goal of in vitro testing is the prediction of the clinical outcome of therapy. The use of the M27-A and M38-A procedures that were developed by the National Committee for Clinical Laboratory Standards (NCCLS) has led to increased inter-laboratory agreement of minimum inhibitory concentrations (MICs). Reproducibility of MICs for yeasts has facilitated the establishment of interpretive breakpoints for fluconazole, itraconazole, and ketoconazole, and Candida spp. This paper reviews the advantages and disadvantages of the available methods for antifungal susceptibility testing of yeasts and moulds as well as the clinical implications of in vitro antifungal testing.

NCCLS Methods for Yeasts Broth Macro- and Micro-Dilution Methods

Eleven systemic antifungal agents are currently licensed for treatment of systemic mycoses: the polyene amphotericin B and its 3 lipid formulations, the imidazoles miconazole and ketoconazole, the triazoles fluconazole, itraconazole, and voriconazole, the pyrimidine synthesis inhibitor flucytosine (5-FC), and the glucan synthesis inhibitor (echinocandin) caspofungin. New triazoles, other echinocandins and glucan synthesis inhibitors are under clinical evaluation. In 1997, the NCCLS published an approved reference method (M27-A document) for the in vitro testing of 5 systemic antifungal agents against Candida spp. and Cryptococcus neoformans. The NCCLS document describes a broth macrodilution method and its microdilution modifications, specifies a defined culture medium as the standard test medium (RPMI-1640 broth buffered to pH 7.0 with MOPS), as well as an inoculum standardized by spectrophotometric reading to approximately 1,000 cells/mL, and the visual determination of the MIC endpoint after incubation at 35°C for 48 (Candida spp.) to 72 hours (C. neoformans).

For the microdilution method, fucytosine and azole MICs are defined as the lowest drug concentrations at which a “prominent decrease in turbidity” is observed as compared with the growth in the control (drug-free medium). However, it has been reported that the specified prominent decrease in turbidity or growth corresponds to approximately 50% reduction in growth as compared with the corresponding growth control. For the macrodilution method, the endpoint is the lowest concentration at which growth is reduced to 20% of the growth control (80% inhibition). For amphotericin B, the endpoint is defined as the lowest drug concentration at which no growth is visible by both methods. The different endpoint criteria for the 2 M27-A methods for azole testing can be confusing and have been used indiscriminately. In the interest of producing reproducible MICs, it is recommended that MICs should be determined by following the corresponding criterion for MIC endpoint determination for each test as described in the NCCLS M27-A document.

M27-A Medium Modifications

In response to specific problems regarding the performance of the standard medium, 3 medium modifications have been highlighted in the M27-A document. (i) Yeast nitrogen base (YNB) medium enhances the growth of C. neoformans, which facilitates the determination of MICs and may improve the clinical relevance of antifungal MICs. (ii) The difference in amphotericin B MICs between susceptible and resistant strains is more pronounced when antibiotic medium 3 (AM3) is used instead of the standard RPMI medium. However, complete separation of resistant from susceptible isolates is not always achieved by using AM3 medium. This problem was reported recently for Candida spp. isolates recovered from patients with candidemia who were failing amphotericin B therapy. Because AM3 is a non-standardized medium and lot-to-lot variation is a limiting factor to its use, it is recommended to introduce as control susceptible and resistant ATCC yeast strains for which the amphotericin B MICs are known. (iii) Increasing the glucose concentration of the standard RPMI medium from 0.2% to 2% results in better growth of most yeasts and may simplify MIC determination. Multi-center studies must assess the reproducibility of MIC data obtained by employing this modified RPMI.
Trailing Growth and the Incubation Time

The term ‘trailing’ has been used to describe the reduced persistent growth or turbidity which some isolates of *Candida* spp. exhibit above the azole MIC. Trailing precludes an easy and reproducible MIC determination; in some cases, agitation of the microdilution trays before MICs are determined may facilitate their interpretation. In other cases, MICs for these trailing isolates are much higher at 48 than at 24 hours, because the trailing growth is heavier at 48 hours.11,12 These isolates would be classed as resistant to fluconazole at 48 hours (>64 µg/mL), but growing evidence suggests that they are susceptible (<8 µg/mL). Ten *Candida albicans* which exhibited trailing growth in fluconazole were found susceptible by sterol quantitation and the standard method at 24 hours, but resistant by the standard method at 48 hours.13,14 Moreover, most episodes of oral candidiasis in HIV-infected individuals, in which isolates with trailing endpoints have been recovered, have responded to low doses of fluconazole.12 One potential solution to the trailing problem is lowering the pH of the standard medium to 5.0,15 which can reduce trailing without affecting fluconazole MICs for susceptible or resistant *C. albicans* isolates to this agent. Further studies must determine if this modification affects the MICs of other antifungal agents and yeast species.

Quality Control (QC)

Quality control of MIC data is essential to ensure the reproducibility of the results. A recent multicenter study has established QC MIC ranges for the microdilution method at both 24 and 48 hours with the established agents, 3 investigational triazoles and 2 echinocandins.16

Broth-Based Alternative Approaches

The NCCLS reference procedures rely upon the visual determination of MIC endpoints.1 Various modifications to the reference procedure have been suggested to facilitate and increase the precision of endpoint determination as discussed below.

Colorimetric Antifungal Panels

Commercial colorimetric panels for the in vitro testing of antifungal agents are available for investigational purposes. Comparisons of the Sensititre Yeast One Panel (Trek Diagnostic Systems, Westlake, OH), that incorporates the indicator Alamar Blue as the colorimetric indicator, with the NCCLS reference procedures have demonstrated close agreement between the methods.17,18 Similar results were obtained during the evaluation of the commercial ASTY colorimetric panel (Kyokuto Pharmaceutical Industrial M, Tokyo, Japan) for testing *Candida* spp.19 A recent multicenter comparison of colorimetric MICs by the Yeast One Panel and the NCCLS microdilution method for >1,000 yeasts has demonstrated that fluconazole and azole MICs should be read at 24 hours and amphotericin B MICs at 48 hours by the Yeast One Panel.18 This panel has been approved by FDA for testing fluconazole, fluconazole, and itraconazole.

Fungitester

Another simpler alternative to the NCCLS reference procedures is breakpoint testing, in which the growth of isolates is measured in cultures containing just 1 or 2 drug concentrations that distinguish resistant from susceptible strains. Comparisons of the Fungitester panel (Sanofi Diagnostics Pasteur M, Paris, France) with the NCCLS broth microdilution method have demonstrated good agreement between the methods forazole-susceptible strains of *Candida* spp., but have indicated that the breakpoint procedure often fails to detect azole-resistant strains.20,21

Spectrophotometric Method

The determination of spectrophotometric MICs requires the selection of a level of inhibition. The best agreement with visual NCCLS endpoints has been obtained with 80% or 90% growth inhibition spectrophotometric endpoints22,23 for amphotericin B and 50% for fluconazole and the azoles.9,22 These inhibition endpoints have also been employed for the spectrophotometric determination of MICs of caspofungin, anidulafungin (90%), and the new triazoles posaconazole and voriconazole (50%).2,3 In addition, Rex and colleagues11 demonstrated that 50% inhibition spectrophotometric endpoints, determined after 24 hours incubation, showed the best correlation with outcome of fluconazole treatment in a murine model of candidiasis. Further studies should clarify this issue.

Ghammoum and colleagues24 developed a modified broth microdilution method for *C. neoformans* using YNB medium supplemented with 0.5% glucose buffered to pH 7.0, an inoculum of 10,000 cells/mL, and incubation at 35°C for 48 hours. The endpoint is determined with a spectrophotometer and is defined as the lowest drug concentration that reduces growth to 50% of control growth. This method gives comparable fluconazole results for *C. neoformans* to those obtained by the NCCLS procedure.25 However, further evaluations should ensure that this method is superior to the standard method for the susceptibility testing of *C. neoformans* to other antifungal agents.

Agar-Based Alternative Methods

The NCCLS procedures1 are time-consuming and cumbersome to perform. In addition to the commercial methods discussed, other simpler and more economic methods for routine clinical use have been evaluated as discussed below.

Disc Diffusion Methods

Although agar disc diffusion has had limited application in antifungal drug susceptibility testing, it has proved useful for in vitro testing of fluconazole. Several investigators26,27 compared agar disc diffusion methods with the NCCLS broth macrodilution method and showed that although the simpler test gave unequivocal results for fluconazole-susceptible strains of *Candida* spp., it could not be relied upon to distinguish resistant isolates (>64 µg/mL MICs) from those with susceptible dose-dependent MICs (16 to 32 µg/mL). Lozano-Chiu and colleagues28 described a method for the determination of susceptibilities of *Candida* spp. to caspofungin. Clearly, disc
diffusion has the potential to provide a simple means of performing in vitro tests with fluconazole and other antifungal agents, but studies including larger number of Candida spp. isolates and other antifungal agents are needed.

**Etest**

The Etest (AB Biodisk M, Solna, Sweden) is a commercially available method (for investigational purposes only in the United States) for determination of MICs for Candida spp. and C. neoformans. It is set up in a manner similar to a disc diffusion test, but the disc is replaced with a calibrated plastic strip impregnated with a continuous concentration gradient of the antimicrobial agent. Comparisons of the MICs obtained with NCCLS procedures and the Etest have shown different levels of agreement, but the agreement is species and medium dependent.29-33 Solidified RPMI medium with 2% dextrose provides the best performance for Etest MICs. Etest is superior to the NCCLS broth macrodilution method for distinguishing amphotericin-B-resistant and susceptible Candida spp. isolates.34,35 Amphotericin B MICs of ≥0.38 µg/mL obtained by Etest have been associated with therapeutic failure for candidemia.36

**Fungicidal Endpoints**

Standard testing guidelines are not available for the evaluation of the in vitro fungicidal activity of antifungal agents. One of the reasons is that most established agents, like the azoles, have only fungistatic or inhibitory activity against yeasts. The determination of minimum fungicidal or lethal concentrations (MFCs or MLCs) involve the subculturing of fixed volumes from each MIC tube or well that shows complete growth inhibition of growth onto an agar. The criteria for MFC determination vary in different publications and the MFC has been described as the lowest drug concentration resulting in either no growth or 3 to 5 colonies.3,4 It has been reported that amphotericin B MFCs were better predictors of microbiologic failure (defined as persistence of Candida in bloodstream despite ≥3 days of amphotericin B) in candidemia infections than conventional MICs with AM3 medium.3 The clinical relevance of the MFC value as well as the development of standard guidelines for MFC determination should be addressed.

**NCCLS Methods for Moulds**

**Broth Macro- and Micro-Dilution Methods**

The development of the standard methodology for yeasts led to the identification of various standard guidelines for mould antifungal susceptibility testing. Early multicenter studies demonstrated that reliable non-germinated conidia or sporangiospore suspensions could be standardized by a spectrophotometric procedure and that test inocula of approximately 10,000 cells/mL provided the most reproducible data.37-40 Based on these results, the NCCLS M38-A document was published for testing moulds that are more frequently obtained with NCCLS procedures and the Etest have shown different levels of agreement, but the agreement is species and medium dependent.29-33 Solidified RPMI medium with 2% dextrose provides the best performance for Etest MICs. Etest is superior to the NCCLS broth macrodilution method for distinguishing amphotericin-B-resistant and susceptible Candida spp. isolates.34,35 Amphotericin B MICs of ≥0.38 µg/mL obtained by Etest have been associated with therapeutic failure for candidemia.36

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**Broth-Based Alternative Approaches**

**Evaluation of Morphologic Changes**

The trailing effect is not a problem when testing either investigational or established azoles against most moulds. However, when testing the echinocandins by broth-based assays and employing the conventional criterion of MIC determination, most Aspergillus isolates would be categorized as resistant, because trailing growth is present in all MIC wells. It was reported by Kurtz and colleagues45 that a more careful visual examination of the trailing observed with an echinocandin against Aspergillus would reveal the presence of very compact, round microcolonies or “clumps” in these wells. Under microscopic examination, these macroscopic clumps corresponded to significant morphologic alterations as the hyphae grew abnormally as short, highly branched filaments with swollen germ tubes and distended, balloon-like cells. Kurtz and colleagues45 reported these morphologic changes as minimum effective concentrations (MECs) to distinguish them from traditional MICs. The reliability and clinical significance of MEC endpoints should be established.

**Colorimetric Method**

It was demonstrated that determination of colorimetric MICs enhanced the inter-laboratory agreement of itraconazole MICs.38,41 This is achieved by adding the colorimetric indicator Alamar Blue (2X, Trek Diagnostics) to 2X concentrated standard RPMI broth, and by following
the steps described in the M38-A document. After the incubation times, wells are examined for a change in color from blue (indicating no growth) to purple (indicating partial inhibition) or to red (indicating growth). The colorimetric MIC of the azoles is the drug concentration that shows a slight change of color from blue to purple; for amphotericin B, the drug concentration that shows no color change or the first well that remains blue.38,41

Agar-Based Alternative Methods
Like the yeasts, agar-based antifungal susceptibility methods have been applied to test the susceptibilities of moulds to different antifungals. More recently, variations of previous agar dilution and disc diffusion methods have been investigated for mould testing44,46 as well as Etest.47,48

Agar Dilution Methods
Agar dilution methods involve the preparation of doubling dilutions (10 times the desired strength) of the agent being evaluated, which are incorporated into molten agar. The prepared drug-containing agar plates are inoculated with inoculum suspensions that could range from 100,000 to 10,000,000 cells/mL.

Disc Diffusion Methods
By using paper discs impregnated with either caspofungin or amphotericin B, Bartizal and colleagues46 were able to obtain critical concentration values (CC values) that correlated better than the conventional MIC endpoint with in vivo data for caspofungin.

Etest
The Etest method has also been adapted for the testing of opportunistic mould pathogens.47,48 Since the trailing growth effect is not a major problem for azole testing against most moulds, Etest inhibition ellipses for the moulds are usually sharper, and MICs are easily interpreted. Overall, comparisons of Etest with NCCLS methods for the moulds have demonstrated better agreement when testing itraconazole (>90%) than amphotericin B (>80%).47,48 Etest MICs of amphotericin B for certain species (A. flavus, P. boydii, Scedosporium prolificans) were usually substantially higher than NCCLS MICs; this phenomenon was more evident with Etest MICs for A. flavus at 48 hours.48 It is noteworthy that infections caused by these fungal species are usually more refractory to antifungal therapy. The reliability and the clinical relevance of Etest MICs should be addressed.

Fungicidal Antifungal Activity
Like yeast testing, standard guidelines are not available for the determination of MFCs. In contrast to the yeasts, the azoles appear to have certain level of fungicidal activity for some opportunistic fungi, including Aspergillus spp. Several studies have examined the fungicidal activity of different species of Aspergillus spp. to amphotericin B,39-54 itraconazole,3-52,54 voriconazole,50-54 and posaconazole.3-49 Three studies have identified the low fungicidal activity of amphotericin B against A. terreus, and that itraconazole50,54 and posaconazole49 appeared to have more fungicidal activity against this species. The poor in vitro fungicidal activity of amphotericin B appears to correspond with the poor absorption and/or patient compliance, rather than azole resistance, can be responsible for clinical failure. Therefore, a low MIC does not necessarily predict clinical success, but a resistant value is less likely associated with response to the corresponding antifungal agent. Isolates of Candida kru sei are resistant to fluconazole regardless of the MIC. Breakpoints also have been established for fluconazole based on historical data and pharmacokinetics of this agent. Breakpoints for other drug-organism combinations have yet to be established.

Clinical Relevance
In order to be clinically useful, in vitro methods should reliably predict in vivo response to therapy. However, drug pharmacokinetics, drug interactions, factors related to the host immune response and/or the status of the current underlying disease, proper patient management, and factors related to the virulence of the infecting organism and its interaction with both the host and the therapeutic agent appear to have more value than the MIC as the predictors of clinical outcome.59 Interpretive MIC breakpoints have been established following correlation with clinical data in oropharyngeal candidiasis (fluconazole and itraconazole) and candidemia in non-neutropenic patients (fluconazole). Isolates inhibited by 64 µg/mL of fluconazole and 1 µg/mL itraconazole are considered susceptible to these agents. Isolates inhibited by ≤8 µg/mL of fluconazole and 0.12 µg/mL of itraconazole are considered susceptible. Fluconazole MICs of 16 to 32 µg/mL and itraconazole MICs of 0.25 to 0.5 µg/mL have been designated as susceptible-dose dependent (S-DD). For fluconazole, this designation comprises isolates for which susceptibility is dependent on achievable peak serum levels of 40 to 60 µg/mL at fluconazole dosages of 800 mg/dL, versus expected peak levels of ≤30 µg/mL at lower dosages. For itraconazole, the S-DD range indicates the need for plasma concentrations in excess of 0.5 µg/mL for an optimal response. Low dosage, poor absorption and/or patient compliance, rather thanazole resistance, can be responsible for clinical failure. Therefore, a low MIC does not necessarily predict clinical success, but a resistant value is less likely associated with response to the corresponding antifungal agent. Isolates of Candida kru sei are resistant to fluconazole regardless of the MIC. Breakpoints also have been established for fluconazole based on historical data and pharmacokinetics of this agent. Breakpoints for other drug-organism combinations have yet to be established.


